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ORIGINAL ARTICLE

Silymarin and silibinin cause G1 and G2–M cell cycle arrest via distinct circuitries in human prostate cancer PC3 cells: a comparison of flavanone silibinin with flavanolignan mixture silymarin

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Here, we assessed and compared the anticancer efficacy and associated mechanisms of silymarin and silibinin in human prostate cancer (PCA) PC3 cells; silymarin is comprised of silibinin and its other stereoisomers, including isosilybin A, isosilybin B, silydianin, silychristin and isosilychristin. Silymarin and silibinin (50–100 μg/ml) inhibited cell proliferation, induced cell death, and caused G1 and G2–M cell cycle arrest in a dose/time-dependent manner. Molecular studies showed that G1 arrest was associated with a decrease in cyclin D1, cyclin D3, cyclin E, cyclin-dependent kinase (CDK)4, CDK6 and CDK2 protein levels, and CDK2 and CDK4 kinase activity, together with an increase in CDK inhibitors (CDKIs) Kip1/p27 and Cip1/p21. Further, both agents caused cytoplasmic sequestration of cyclin D1 and CDK2, contributing to G1 arrest. The G2–M arrest by silibinin and silymarin was associated with decreased levels of cyclin B1, cyclin A, pCdc2 (Tyr15), Cdc2, and an inhibition of Cdc2 kinase activity. Both agents also decreased the levels of Cdc25B and cell division cycle 25C (Cdc25C) phosphatases with an increased phosphorylation of Cdc25C at Ser216 and its translocation from nucleus to the cytoplasm, which was accompanied by an increased binding with 14-3-3β. Both agents also increased checkpoint kinase (Chk)2 phosphorylation at Thr68 and Ser19 sites, which is known to phosphorylate Cdc25C at Ser216 site. Chk2-specific small interfering RNA largely attenuated the silymarin and silibinin-induced G2–M arrest. An increase in the phosphorylation of histone 2AX and ataxia telangiectasia mutated was also observed. These findings indicate that silymarin and silibinin modulate G1 phase cyclins–CDKs–CDKIs for G1 arrest, and the Chk2–Cdc25C–Cdc2/cyclin B1 pathway for G2–M arrest, together with an altered subcellular localization of critical cell cycle regulators. Overall, we observed comparable effects for both silymarin and silibinin at equal concentrations by weight, suggesting that silibinin could be a major cell cycle-inhibitory component in silymarin. However, other silibinin stereoisomers present in silymarin also contribute to its efficacy, and could be of interest for future investigation.
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Keywords: prostate cancer; silibinin; silymarin; cell cycle arrest; cyclin-dependent kinase

Introduction

Prostate cancer (PCA) is a major cancer in the USA and European countries, and the second leading cause of cancer deaths in American men (American Cancer Society, 2005). According to the American Cancer Society, 232 090 new cancer cases and 30 350 deaths are estimated to occur due to PCA in 2005 (American Cancer Society, 2005). The increasing incidences and mortality due to PCA and the failure of conventional chemo- and radio-therapy of advanced invasive PCA indicate that new approaches are essentially needed for the control of this malignancy (Koivisto *et al*., 1998; Feldman and Feldman, 2001). In this regard, prevention and therapeutic intervention by phytochemicals is a newer dimension in cancer management. Administration of phytochemicals is shown to prevent initiational, promotional and progressional events associated with carcinogenesis in different animal models, and is suggested to effectively reduce cancer mortality and morbidity (Surh, 2003). Among various groups of phytochemicals, extensive experimental data have been generated for polyphenolic flavonoids for their role in chemoprevention of various cancers including PCA (Dhanalakshmi *et al*., 2002; Agarwal *et al*., 2003; Hou *et al*., 2004; Neuhouser, 2004).

Silibinin is a polyphenolic flavonoid isolated mainly from the fruits or seeds of milk thistle (*Silybum marianum*), and silymarin is a flavonolignan complex, composed of silibinin and small amounts of its stereoisomers, namely, isosilybin A, isosilybin B, silychristin, isosilychristin and silydianin (Wagner *et al*., 1974). Silibinin was recently shown to be a 1:1 mixture of the

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stereoisomers, silybin A and silybin B, and constitutes about 40% (w/w) of silymarin (Kim *et al*., 2003). Both silibinin and silymarin have human consumption and acceptability data, and have been in clinical use for over three decades in Europe, and recently in Asia and the United States (Wellington and Jarvis, 2001). Silibinin/silymarin is well known for its hepatoprotective activity, and is reported to protect from liver injury in animal models caused by intoxicants such as carbon tetrachloride, galactosamine, thioacetamide, ethanol, paracetamol (acetaminophen), benzo[*a*]pyrene, thallium, α-amanitin, bacterial endotoxins, γ-radiation, etc., as well as in humans (Flora *et al*., 1998; Pares *et al*., 1998; Wellington and Jarvis, 2001). Several studies by others and us in last 10 years have suggested that silibinin and silymarin possess anticancer potential against many epithelial cancers including PCA (Zi *et al*., 1998a; Bhatia *et al*., 1999; Sharma *et al*., 2003; Mallikarjuna *et al*., 2004; Singh and Agarwal, 2004; Singh RP *et al*., 2004; Thelen *et al*., 2004). However, its mechanism/s of action is/are not known completely. In the present study, for the first time we assessed the anticancer efficacy targeting cell cycle regulation and associated molecular mechanisms of pure compound silibinin in human PCA PC3 cells, and compared with silymarin, which is the major flavanolignan complex present in dietary supplement milk thistle extract.

Several studies have demonstrated a close association between deregulation of cell cycle progression and development of cancer, and suggested that inhibition of unchecked cell cycle regulation in cancer cells could be a potential target for the management of cancer (McDonald and El-Deiry, 2000; Owa *et al*., 2001). The regulation of cell cycle is controlled, in part, by a family of cyclin/cyclin-dependent kinase (CDK) complexes and the CDK inhibitors (CDKI) (Kastan *et al*., 1995; Agarwal *et al*., 2003). G1–S transition is positively controlled by CDK4, 6 and 2 in association with D-type cyclins for CDK4 and 6, and cyclin E and A for CDK2 (Grana and Reddy, 1995; Morgan, 1995; Sherr and Roberts, 1999; Yim *et al*., 2005). These CDK–cyclin complexes are known to phosphorylate the retinoblastoma family of proteins to release E2F transcription factors needed to increase the transcripts for growth responsive genes (Sherr, 1995). Further, CDK–cyclin complexes are negatively controlled by the Kip/Cip family of CDKIs, namely, Kip1/p27 and Cip1/p21, in addition to the INK family of CDKIs (Deng *et al*., 1995; Agarwal *et al*., 2003; Nakayama *et al*., 2004; Takagaki *et al*., 2005). G2–M transition is positively regulated by Cdc2 and cyclin B complex (Taylor and Stark, 2001), and the Cdc25 family of phosphatases regulate the activity of Cdc2 through dephosphorylation of inhibitory phosphorylation at threonine 14 and tyrosine 15, caused by Wee1 or Myt1 (Gautier *et al*., 1991; Sebastian *et al*., 1993). These phosphatases are inactivated through phosphorylation by cellular checkpoint kinases (Chk1/2), which may in turn be activated by upstream kinase ataxia telangiectasia mutated (ATM)-related kinase (ATM/ATR) in response to DNA damage (Zhao *et al*., 2002; Singh SV *et al*., 2004).

In the present study, we observed that silibinin- and silymarin-caused growth inhibition in human PCA PC3 cells is associated with an induction of G1 arrest and G2–M cell cycle arrest. Mechanistic investigation suggests an increase in CDKIs (Kip1/p27 and Cip1/p21) with a concomitant decrease in CDKs and cyclin levels and associated kinase activities leading to G1 arrest. Further, for the first time we report that silibinin and silymarin modulate the ATM–Chk1/2–Cdc25–Cdc2–cyclin B1 pathway for G2–M arrest in PC3 cells. We also observed that these agents alter the cytoplasmic versus nuclear localization of CDKs, cyclins and Cdc to control their activity. Furthermore, we observed almost comparable effects with both silymarin and silibinin when used at equal doses by weight, suggesting the possible contribution of other silibinin stereoisomers present in silymarin for its efficacy.

Results

Silymarin and silibinin inhibit cell proliferation and cause cell death of human prostate carcinoma PC3 cells

First, we examined the dose– and time–response effects of these agents on PC3 cell growth and death. Cells were treated with equal (by weight) concentrations of the agents (50 and 100 μg/ml, final concentration in medium) dissolved in DMSO (vehicle) for 24 and 48 h. At the end of each treatment time, determination of total cell number as well as dead cells showed that both silymarin and silibinin inhibit cell growth and cause cell death in a dose- as well as time-dependent manner (Figure 1a and b). The lower dose of silymarin (50 μg/ml) showed 18 ($P<0.05$) and 26% ($P<0.05$) decreases in total cell number after 24 and 48 h of treatments, respectively (Figure 1a), but its higher dose (100 μg/ml) decreased the cell number by 47% ($P<0.001$) and 59% ($P<0.001$) after 24 and 48 h of treatments, respectively (Figure 1a). Similarly, the treatment with lower dose of silibinin (50 μg/ml) for 24 and 48 h decreased the cell number by 21% ($P<0.005$) and 44% ($P<0.001$), and at higher dose (100 μg/ml) by 51% ($P<0.001$) and 61% ($P<0.001$), respectively (Figure 1a). Using the trypan blue dye exclusion method, we observed that the decrease in cell number by both the agents is accompanied by an increase in cell death. The lower dose of silymarin increased cell death by 1.65 ($P<0.005$) and 2.33 ($P<0.05$) fold, and the higher dose by 2.43 ($P<0.001$) and 5.86 ($P<0.01$) fold after 24 and 48 h of treatments as compared to their respective controls, respectively (Figure 1b). Similarly, lower dose of silibinin increased the cell death by 2.31 ($P<0.005$) and 3.38 ($P<0.005$) fold, and the higher dose by 4.60 ($P<0.001$) and 5.23 ($P<0.001$) fold after 24 and 48 h of treatments as compared to their respective controls (Figure 1b). However, in none of the treatments did the percent cell death increase beyond 10%, suggesting that the overall decrease in cell number only partly contributed to the cell-death-inducing effect of both the agents in PC3 cells. Therefore, we next investigated the effect of silymarin and silibinin on

cell cycle progression to determine whether the inhibitory effect on cell proliferation is accompanied by modulation of cell cycle progression.

Silymarin and silibinin cause G1 and G2–M phase arrest in cell cycle progression of PC3 cells

After desired treatments with silymarin and silibinin, PC3 cells were stained with saponin/propidium iodide (PI) and analysed by flow cytometry. Cell cycle distribution analysis showed that silymarin treatment for 24 h results in a significant increase in G1-phase cells at both the doses ($P<0.001$), along with a dose-dependent increase in G2–M-phase cells ($P<0.01$–0.001), at the expense of a significant decrease ($P<0.001$) in S-phase cells (Table 1). A similar increase in G1 phase cells was observed at 48 h of silymarin treatment ($P<0.001$), but the G2–M phase cells increased only with higher dose at this treatment time ($P<0.001$) (Table 1). Silibinin treatment for 24 h also resulted in a significant increase ($P<0.001$) in G1-phase cells only at lower dose, while G2–M-phase cells increased ($P<0.05$–0.001) at both the doses (Table 1). After 48 h silibinin treatment, the increase in G1-phase cells became significant ($P<0.05$–0.001) at both the doses even though the increase was marginal at the higher dose; however, the G2–M-phase cells increased ($P<0.001$) only at a higher dose of silibinin (Table 1). Together, these results suggest that both silymarin and silibinin cause G1 as well as G2–M arrest in PC3 cells, which were accompanied by a decrease in S-phase cell population. At higher dose, silymarin shows better efficacy for G1 arrest as compared to silibinin, whereas silibinin shows more efficacy for G2–M arrest as compared to silymarin. These data also suggest the presence of bioactive component/s in silymarin other than silibinin for the enhanced and sustained G1 arrest at a higher dose of silymarin. In further studies, we analysed the molecular alterations-associated cell cycle effects of silymarin and silibinin.

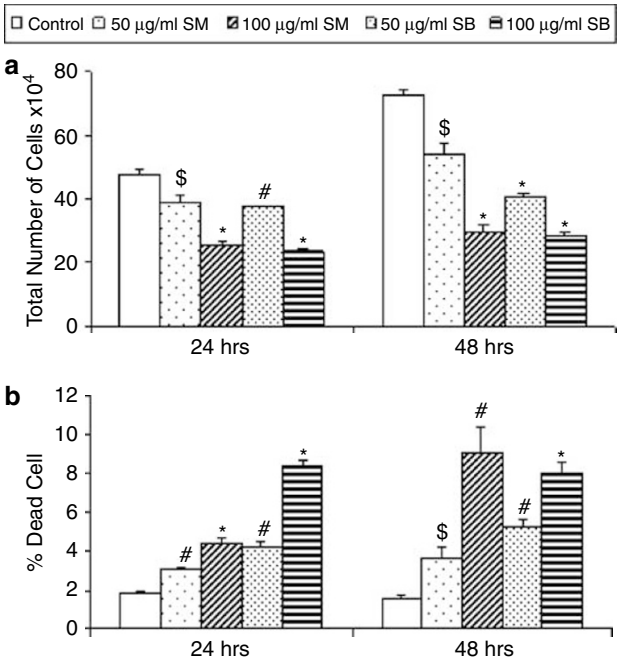

Figure 1 Effect of silymarin and silibinin on cell growth and death in human prostate carcinoma PC3 cells. Cells were treated with DMSO (control) or 50 and 100 μg/ml doses of silymarin or silibinin for 24 and 48 h. At the end of each treatment time, both floaters and attached cells were collected and processed for (**a**) determination of total cell number and (**b**) dead cells as mentioned in 'Materials and methods'. The data shown are mean$\pm$s.e.m. of three samples for each treatment. These results were similar in three independent experiments. SM, silymarin; SB, silibinin; $^{\$}P<0.05$; $^{\#}P<0.01$; $^{*}P<0.001$.

Silymarin and silibinin modulate G1 cell cycle regulators and decrease the activity of CDKs

As progression through the cell cycle is mediated by CDKs complexed with corresponding cyclins (Kastan *et al*., 1995), we next analysed whether silymarin and silibinin modulate the protein levels of G1 CDKs and cyclins in PC3 cells. Western blot analysis showed that silymarin and silibinin treatment for 24 and 48 h results in a moderate to strong decrease in the protein levels of CDK4, CDK2, cyclin D1, cyclin D3 and cyclin E in a dose-dependent manner (Figure 2a and b). A moderate decrease in CDK6 and Cdc25A protein levels was also observed after 48 h of treatment. Since cyclin D1 in association with CDK4 and CDK6, and CDK2 in association with cyclin E play important roles in cell cycle progression through the G1 phase, we next performed a time-kinetics study to analyse the effect of (100 μg/ml) silymarin and silibinin on cyclin D1 and CDK2 protein levels starting from 1 to 48 h. Silymarin

Table 1 Effect of silymarin and silibinin on cell cycle distribution in human prostate carcinoma PC3 cells

Treatment	*24 h*			*48 h*		
	G1	*S*	*G2/M*	*G1*	*S*	*G2/M*
Control	40.3$\pm$1.72	42.3$\pm$1.34	17.4$\pm$0.71	47.1$\pm$0.91	32.3$\pm$0.32	20.6$\pm$0.83
50 μg/ml SM	59.5$\pm$0.29*	19.0$\pm$0.07*	21.5$\pm$0.35$^{\$}$	69.3$\pm$1.83*	11.8$\pm$1.23*	18.9$\pm$0.66
100 μg/ml SM	55.9$\pm$0.11*	17.3$\pm$0.39*	26.8$\pm$0.48*	65.1$\pm$0.67*	7.0$\pm$0.79*	28.0$\pm$0.37*
50 μg/ml SB	61.6$\pm$0.33*	16.4$\pm$0.30*	22.0$\pm$0.32$^{\#}$	70.6$\pm$0.32*	10.2$\pm$0.35*	19.2$\pm$0.12
100 μg/ml SB	43.5$\pm$1.26	21.7$\pm$0.62*	34.9$\pm$1.82*	50.6$\pm$0.58$^{\$}$	14.8$\pm$1.05*	34.6$\pm$0.80*

Data are presented as mean$\pm$s.e.m. ($n=3$) of percent cell population in different phases of cell cycle. SM, silymarin; SB, silibinin; $^{\#}P<0.05$; $^{\$}P<0.01$; $^{*}P<0.001$.

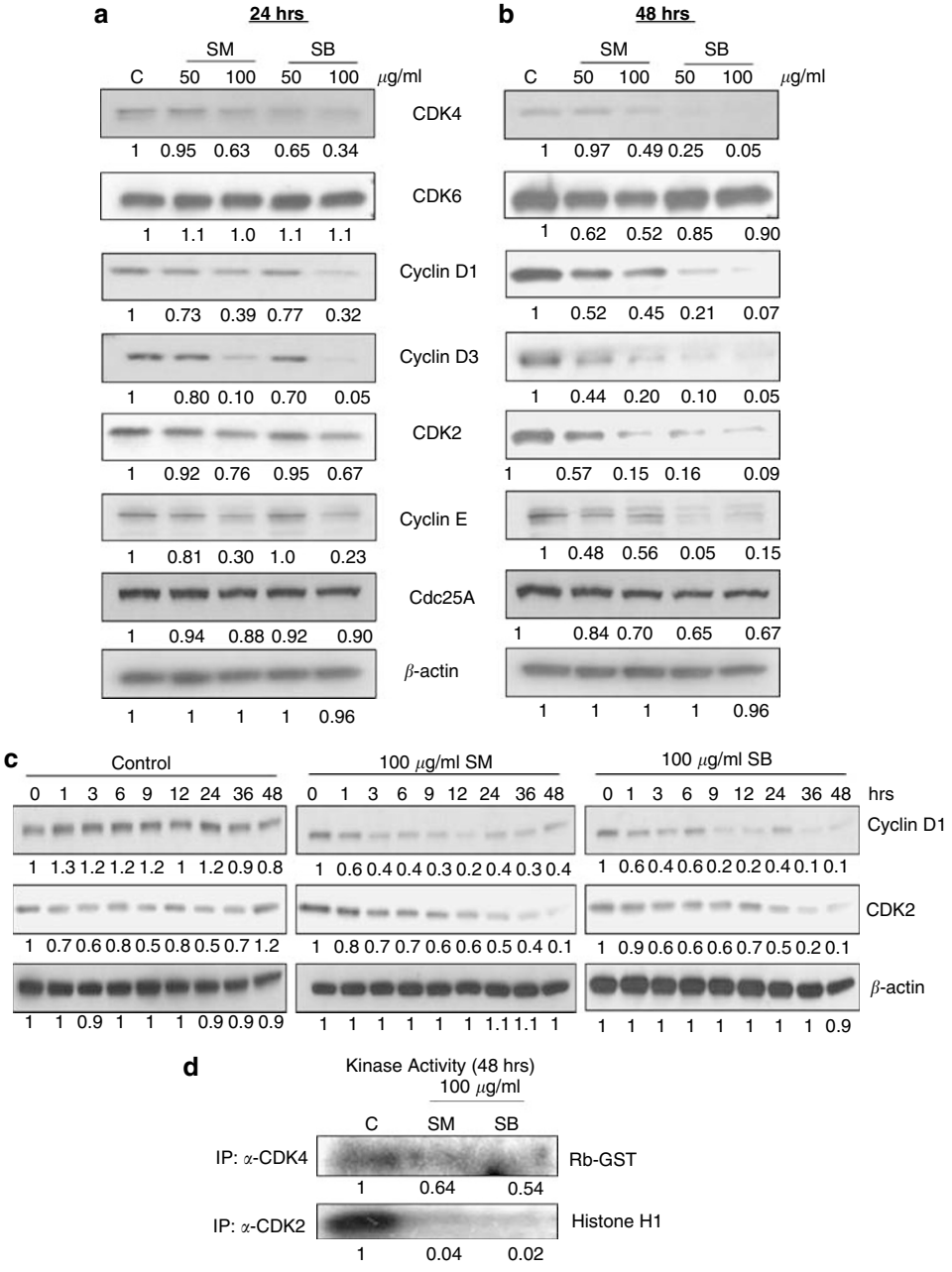

Figure 2 Effect of silymarin and silibinin on G1 phase cell cycle regulators in PC3 cells. (**a**, **b**) Cells were treated with DMSO or 50 and 100 μg/ml doses of silymarin or silibinin for 24 and 48 h. At the end of each treatment time, cell lysates were prepared in nondenaturing lysis buffer as mentioned in 'Materials and methods'. For each sample, 50–60 μg of protein lysate was used for SDS–PAGE and Western immunoblotting, and membranes were probed for CDK4, CDK6, cyclin D1, cyclin D3, CDK2, cyclin E and Cdc25A. Membranes were also stripped and reprobed with anti-β-actin antibody for protein loading correction. (**c**) A time–kinetics study (0–48 h) for the effect of 100 μg/ml dose of silymarin or silibinin on cyclin D1 and CDK2 protein levels analysed in cell lysate by Western immunoblotting. (**d**) Kinase activity of CDK4 for Rb-GST and CDK2 for histone H1 after 48 h of treatment with 100 μg/ml dose of silymarin or silibinin. In-bead kinase assays were performed after immunoprecipitation of the specific protein as described in 'Materials and methods'. In each case, blots shown are representive of three independent experiments. C, control; SM, silymarin; SB, silibinin; IP, immunoprecipitation.

and silibinin caused a decrease in cyclin D1 after 1 h of the treatment, whereas the decrease in CDK2 level was noticeable after 3 h of similar treatment and became more prominent thereafter (Figure 2c). Reprobing of the membranes with anti-β-actin antibody confirmed equal protein loading in each case. These results suggest the possible early inhibitory effect of these agents on cyclin D1-mediated cell cycle progression (early G1) followed by that via CDK2-mediated cell cycle progression (late G1 and G1–S transition) in PC3 cells. We next investigated the effect of silymarin and silibinin on CDK4 and CDK2 kinase activity. The treatment with

silymarin and silibinin (100 μg/ml) for 48 h resulted in a strong decrease in Rb (retinoblastoma)- and histone H1-associated CDK4 and CDK2 kinase activities, respectively, indicating them as possible molecular targets mediating G1 arrest by these agents (Figure 2d).

Effect of silymarin and silibinin on nuclear/cytoplasmic distribution of cyclin D1 and CDK2

Cytoplasmic sequestration of cyclin D1 and CDK2 from the nucleus has been suggested to play an important role in G1 arrest by reducing their activity for the activation of nuclear proteins (Sumrejkanchanakij *et al*., 2003; Yang and Burnstein, 2003). Consistent with these reports and our results showing a G1 arrest by these agents, silymarin and silibinin treatment at 100 μg/ml dose resulted in a stronger decrease in the level of cyclin D1 in nuclear extracts compared to that in cytoplasmic extracts at 24 h of the treatment (Figure 3a), which became more profound after 48 h of similar treatments (Figure 3b). The changes in cytoplasmic versus nuclear levels of CDK2 were not that remarkable after 24 h of the treatment (Figure 3a); however, a strong effect was observed towards a decrease in nuclear CDK2 level as compared to that in cytoplasmic extracts after 48 h of silymarin and silibinin treatments (Figure 3b). These results suggest that silymarin and silibinin treatment not only decreases the total protein levels of cyclin D1 and CDK2 but also alter their cytoplasmic and nuclear localization in order to control their activities.

Effect of silymarin and silibinin on G2–M regulators

Since silymarin and silibinin treatment also resulted in G2–M arrest in PC3 cells (Table 1), we next assessed their effect on the expression of proteins that are associated with the regulation of G2–M transition. Cdc25 phosphatases play a critical role in regulation of cell cycle by dephosphorylation and activation of CDKs (such as CDK1 or Cdc2) at positions Thr14 and Tyr15 (Gautier *et al*., 1991; Sebastian *et al*., 1993). Three human Cdc25 homologs exist: Cdc25A, Cdc25B and cell division cycle 25C (Cdc25C) (Lyon *et al*., 2002). Cdc25A is involved in G1–S phase transition (Hoffman *et al*., 1994), whereas Cdc25B and/or Cdc25C are shown to be necessary for the G2–M transition (Nilsson and Hoffmann, 2000; Turowski *et al*., 2003). The treatment of cells with silymarin and silibinin resulted in a dose-dependent decrease in the levels of Cdc25B and Cdc25C (Figure 4a and b). Moreover, Western blot analysis

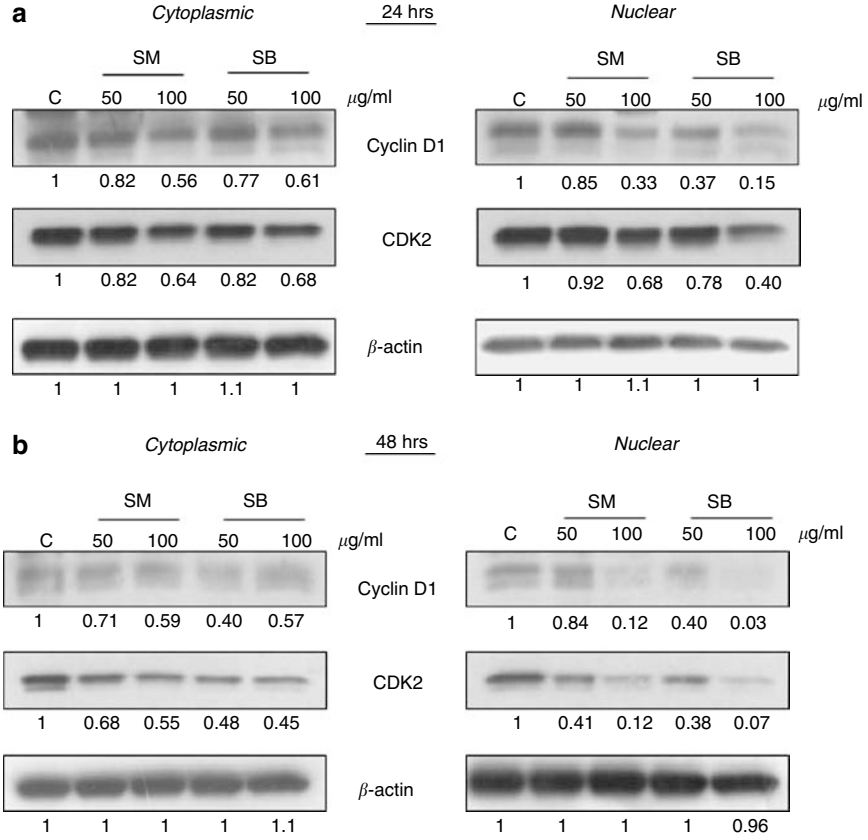

Figure 3 Effect of silymarin and silibinin on nuclear and cytoplasmic distribution of cyclin D1 and CDK2 in PC3 cells. Cells were treated with indicated doses of silymarin or silibinin for (**a**) 24 and (**b**) 48 h, and cytoplasmic and nuclear extracts were prepared as mentioned in 'Materials and methods'. Both cytoplasmic and nuclear extracts were analysed for cyclin D1and CDK2 protein levels by Western immunoblotting as mentioned in 'Materials and methods'. Membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. Blots shown are representive of two independent experiments in each case. C, control; SM, silymarin; SB, silibinin.

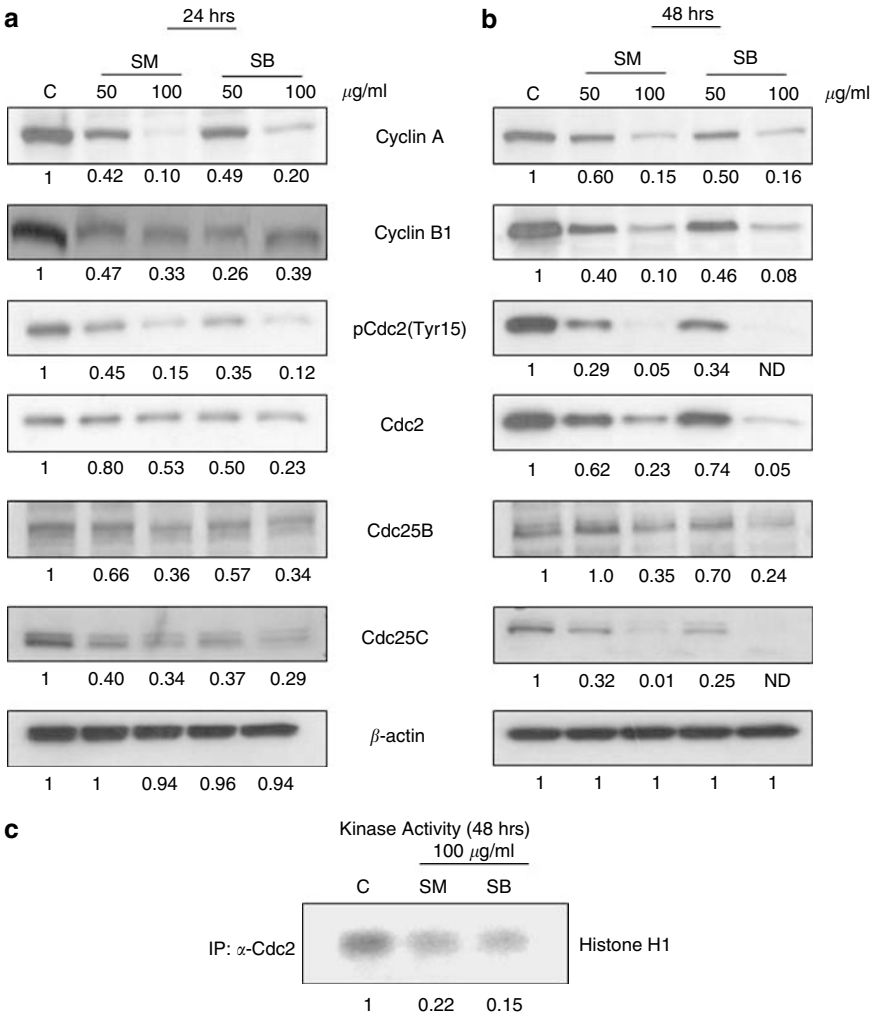

Figure 4 Effect of silymarin and silibinin on G2–M cell cycle regulators in PC3 cells. Cells were treated with DMSO or 50 and/or 100 μg/ml doses of silymarin and silibinin for 24 and/or 48 h. At the end of each treatment time, cell lysates were prepared in nondenaturing lysis buffer as mentioned in 'Materials and methods'. (**a, b**) For each sample, 60 μg of protein lysates was resolved on tris-glycine gel, followed by Western immunoblotting as mentioned in 'Materials and methods'. Membranes were probed for cyclin A, cyclin B1, pCdc2(Tyr15), Cdc2, Cdc25B and Cdc25C protein levels. Membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. (**c**) Cdc2-associated kinase activity for histone H1 was measured by immunoprecipitating Cdc2 and in-bead kinase assay as described in 'Materials and methods'. Blots shown are representive of three independent experiments in each case. C, control; SM, silymarin; SB, silibinin; ND, not detectable.

showed that silymarin and silibinin treatments for 24 and 48 h cause a decrease in the levels of cyclin B1, cyclin A, pCdc2(Tyr15) and Cdc2, and this effect was very prominent at the higher dose and in most cases after 48 h of the treatment (Figure 4a and b). We did not observe any change in the protein level of Wee 1, which is known to phosphorylate Cdc2 (data not shown). In this regard, the decreased level of pCdc2(Tyr15) appears to be due to a decrease in its total protein level. Reprobing of membranes with anti-β-actin antibody confirmed equal protein loading in each case. Consistent with the decrease in these G2–M regulators, as well as induction of G2–M arrest, treatment with 100 μg/ml silymarin and silibinin for 48 h also resulted in a strong decrease in histone H1-associated Cdc2 kinase activity (Figure 4c).

Effect of silymarin and silibinin on CDKIs, Kip1/p27 and Cip1/p21 protein, and Skp2 level

The CDKIs, Kip1/p27 and Cip1/p21 from the Kip/Cip family are known to bind with and inhibit the activity of CDK–cyclin complexes and thus regulate both G1–S and G2–M transitions (Deng *et al*., 1995; Agarwal *et al*., 2003; Nakayama *et al*., 2004; Takagaki *et al*., 2005). Accordingly, next we examined the protein levels of Kip1/p27 and Cip1/p21 by Western blot analysis that showed increased levels of both Kip1/p27 and Cip1/p21 after 24 and 48 h of silymarin and silibinin treatments (Figure 5a and b). The F-box protein S-phase kinase-associated protein 2 (Skp2) is one of the positive regulators of the cell cycle that promotes ubiquitin-mediated proteolysis of CDKIs Kip1/p27 and Cip1/p21 (Bornstein *et al*., 2003; Kossatz *et al*., 2004). Overexpression of

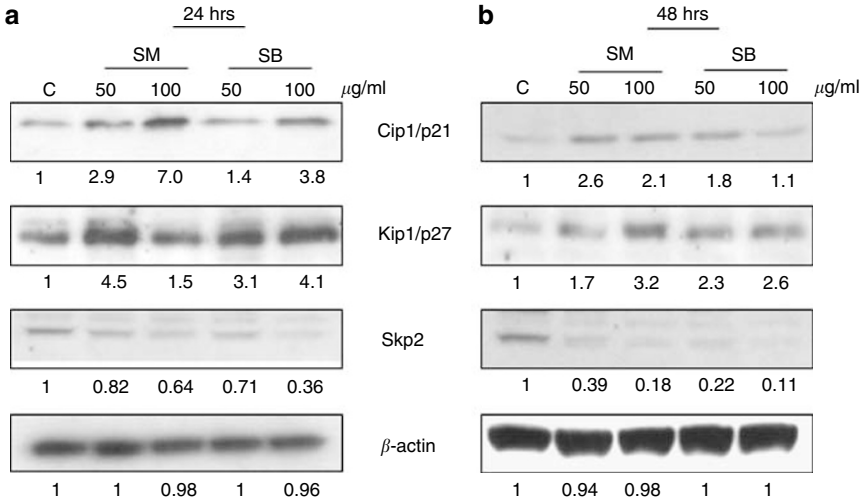

Figure 5 Effect of silymarin and silibinin on CDKIs and Skp2 in PC3 cells. (**a**, **b**) In similar treatments as in Figure 4a and b, cell lysates were analysed for Cip1/p21 and Kip/p27 protein expression by Western immunoblotting as mentioned in 'Materials and methods'. Membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. Blots shown are representive of three independent experiments in each case. C, control; SM, silymarin; SB, silibinin.

Skp2 has been reported in many cancers, including PCA (Gstaiger *et al*., 2001; Li *et al*., 2004; Zheng *et al*., 2004). In further analysis, we observed that silymarin and silibinin strongly decrease the protein level of Skp2 (Figure 5a and b); protein loading in these blots was confirmed by reprobing the same membranes with β-actin antibody as a loading control (Figure 5). These findings suggest that silymarin and silibinin increase the protein levels of Kip1/p27 and Cip1/p21 as one of the possible mechanisms to inhibit CDK–cyclin kinase activity, as well as that the increase in these CDKIs is mediated at least in part by inhibiting their proteosomal degradation due to a decrease in Skp2 as one of the possible mechanisms of their regulation.

Effect of silymarin and silibinin on the level of pCdc2(Tyr15) and its nuclear/cytoplasmic distribution

Since the levels of Cdc25B and Cdc25C phosphatases were reduced markedly with silymarin and silibinin treatment, we expected an increase in phosphorylation of Cdc2 at Tyr15 site, but, surprisingly, the phosphorylation at this site was decreased. To assess whether this decrease in pCdc2(Tyr15) is due to a decrease in its total protein level, we performed a time–kinetics study to measure the changes in the phosphorylation of Cdc2 as well as its total protein level after silymarin and silibinin treatment. The immunoblot results show that decrease in the phosphorylation of Cdc2 at Tyr15 site was in tandem with the decrease in its total protein level (Figure 6a). We, therefore, next examined the nuclear and cytoplasmic localization of pCdc2(Tyr15) and its total protein level after 48 h of silymarin and silibinin treatment at both lower and higher doses. Cdc2 total protein level decreased in both cytoplasmic and nuclear fractions, but the decrease was more remarkable in nuclear fraction, which could not be detected at higher doses (Figure 6b). With regard to phospho-Cdc2(Tyr15), the decrease was more drastic in the cytoplasmic fraction compared to the nuclear fraction, especially at the lower doses of silymarin and silibinin (Figure 6b). Taken together, these results suggest that a decrease in total Cdc2 protein level along with an increase in pCdc2(Tyr15) in nuclear pool of Cdc2 (comparing the ratio of phospho- to total protein in treated samples versus that of control), and a relative decrease in the pCdc2(Tyr15) in cytoplasmic pool of Cdc2, might be associated with the observed decrease in Cdc2 kinase activity contributing to G2–M arrest.

Effect of silymarin and silibinin on phosphorylation of Cdc25C, its binding with 14-3-3β *and nuclear and cytoplasmic localization*

Since we observed a relatively increased ratio of pCdc2(Tyr15)/total Cdc2 in nuclear fraction without any change in upstream Wee1 kinase by silymarin and silibinin treatment, one strong possibility was an involvement of Cdc25C that plays a vital role in G2–M transition (Turowski *et al*., 2003). We, therefore, next assessed whether and how silymarin and silibinin alter the activity of Cdc25C by assessing its phospho- and total protein levels, changes with different treatment time, binding with 14-3-3β and nuclear/cytoplasmic localization. Silymarin and silibinin treatment of cells for 24 and 48 h resulted in a very strong increase in phosphorylation of Cdc25C at Ser216 position; however, there was a strong decrease in its total protein level (Figure 7a and b). In the time–kinetics study (1–48 h), these agents showed a moderate to strong decrease in Cdc25C total protein level starting at 3 h; however, total Cdc25C levels were undetectable after 36 and 48 h of treatment (Figure 7c). In other studies, consistent with earlier reports showing that Cdc25C phosphorylation at serine-216 creates a binding site for 14-3-3β proteins and results in its export to and retention in the cytoplasm in

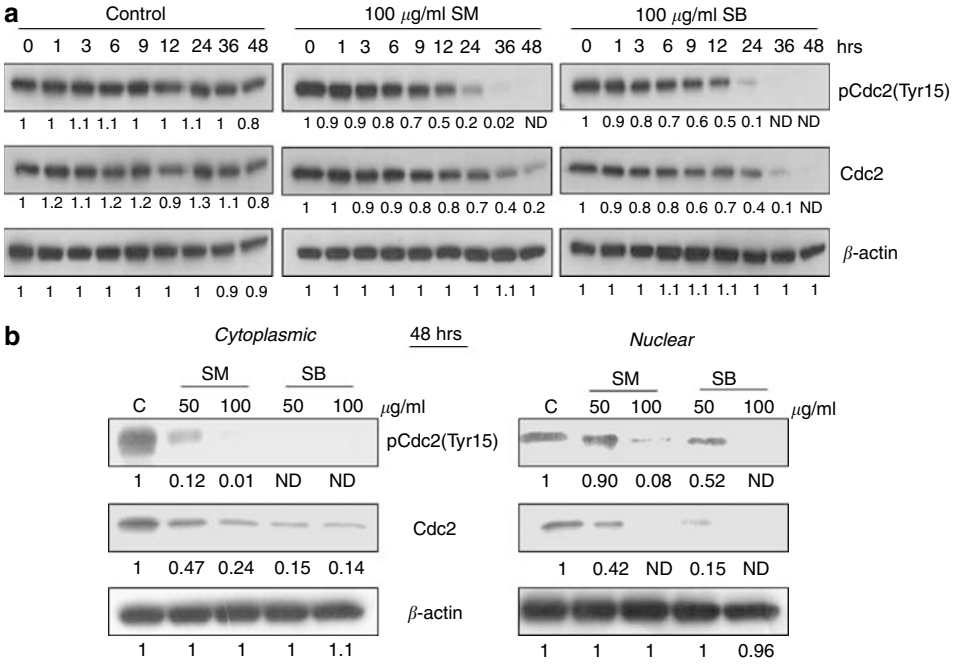

Figure 6 Effect of silymarin and silibinin on the phosphorylation of Cdc2 and its nuclear and cytoplasmic localization in PC3 cells. (**a**) Cells were treated with DMSO or 100 μg/ml dose of silymarin or silibinin for 0–48 h, and cell lysates were prepared at different time intervals as indicated in the figure. Relative changes in the phosphorylation of Cdc2(Tyr15) and its total protein were analysed by Western immunoblotting as mentioned in 'Materials and methods'. (**b**) Cells were treated with DMSO or 50 and 100 μg/ml doses of silymarin or silibinin for 48 h and nuclear and cytoplasmic extracts were prepared as described in 'Materials and methods'. For each sample, 50 μg of protein lysates was used for SDS–PAGE and Western immunoblotting, and membranes were probed for pCdc2(Tyr15) and Cdc2. In each case, membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. Blots shown are representive of three independent experiments in each case. C, control; SM, silymarin; SB, silibinin; ND, not detectable.

the inactive form (Peng *et al*., 1997; Lopez-Girona *et al*., 1999), we observed that these agents increase phosphorylation of Cdc25C at the Ser216 site as early as 6 h of the treatment (Figure 7d), as well as the binding of Cdc25C with 14-3-3β (Figure 7e). In addition, silymarin and silibinin also resulted in a drastic decrease in the nuclear levels of Cdc25C (Figure 8a). Western blot analysis showed an increased level of pCdc25C (Ser216) in the cytoplasmic fraction, which almost vanished in the nuclear fraction (Figure 8a). A decrease in total Cdc25C protein level was also observed in cytoplasmic fraction, but the effect was more prominent in the nuclear fraction following treatment of the cells with these agents (Figure 8a). We further examined Cdc25C localization by immunocytochemistry, where cells were treated with DMSO (control) or with silymarin or silibinin (100 μg/ml) for 48 h and then stained with anti-Cdc25C antibody (green) and nucleic acid-binding dye PI (red). In control cells, Cdc25C (green staining) was distributed in the cytoplasm as well as in the nucleus. Consistent with the western immunoblot analysis data, the nuclear localization of Cdc25C was relatively less as compared to its cytoplasmic localization (Figure 8b). Results also showed that there was an overall decrease in green staining (Cdc25C level) with silymarin and silibinin treatment, which was also in agreement with the result obtained with immunoblot analysis (Figure 8b). Together, these results suggested that the decrease in total Cdc25C protein accompanied by its altered subcellular distribution as well as an increase in its phosphorylation by upstream kinases (such as Chk1/2), could be associated with its decreased phosphatase activity for Cdc2 by silymarin and silibinin.

Chk2 activation plays a critical role in silymarin- and silibinin-induced G2–M arrest in PC3 cells

Chk2 is a potential upstream kinase for the phosphorylation of Cdc25C at Ser216 site, which is ultimately linked to the blockade of cell cycle progression at G2–M phase (Singh SV *et al*., 2004). We examined whether silymarin and silibinin affect the activated form of Chk2 that is usually phosphorylated at Thr68 and/or Ser19 sites. Western blot analysis showed that there was a moderate to strong increase in the phosphorylation of Chk2 at Thr68 and Ser19 sites by both silymarin and silibinin (50 and 100 μg/ml) treatment for 48 h (Figure 9a and b). However, at 24 h of treatment, phospho-Chk2(Thr68) level was increased by only higher dose of silibinin, and phospho-Chk2(Ser19) level by higher dose of silymarin and both doses of silibinin. We did not observe any considerable change in total Chk2 protein level with any of these treatments (Figure 9a and b). To further verify the role of Chk2 in silymarin- and silibinin-induced G2–M cell cycle arrest, we used the siRNA technique to suppress or knock

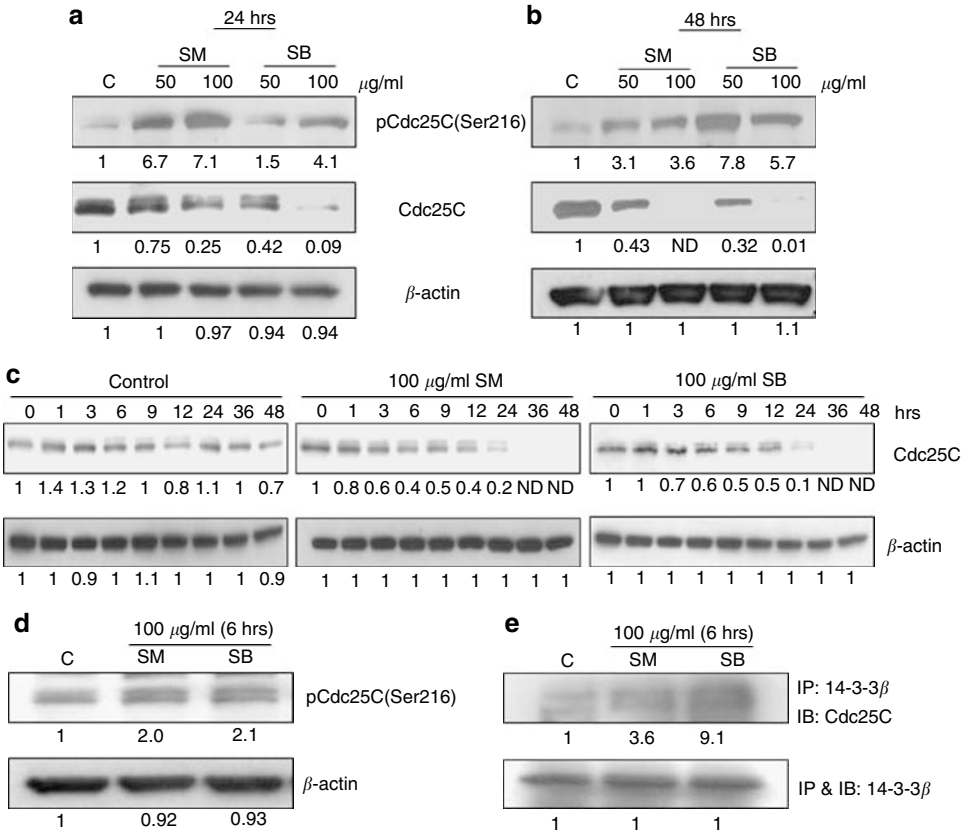

Figure 7 Effect of silymarin and silibinin on Cdc25C(Ser216) phosphorylation and total protein, and its binding with 14-3-3β in PC3 cells. (**a**, **b**) Cells were treated with DMSO or 50 and 100 μg/ml doses of silymarin or silibinin for 24 and 48 h, and cell lysates were prepared and analysed for pCdc25C(Ser216) and Cdc25C protein levels by Western immunoblotting. (**c**) A time–kinetics study was performed for Cdc25C protein levels after similar treatments as in Figure 6a. (**d**) After 6 h of DMSO or 100 μg/ml dose of silymarin or silibinin treatments, cell lysates were analysed for pCdc25C(Ser216), and (**e**) for binding of Cdc25C with 14-3-3β by immunoprecipitation with anti-14-3-3β antibody, followed by immunoblotting with anti-Cdc25C antibody. The same membrane was stripped and probed for 14-3-3β protein levels. (**a**–**d**) Membranes were also stripped and reprobed for β-actin for protein loading correction. Blots shown are representive of at least two independent experiments in each case. C, control; SM, silymarin; SB, silibinin; IP, immunoprecipitation; IB, immunoblotting; ND, not detectable.

down the expression of Chk2 protein. In cell cycle analysis, silymarin and silibinin (100 μg/ml)-induced G2–M arrest was completely reversed ($P<0.005$ and $P<0.003$) by Chk2 siRNA after 24 h of the treatment, and was comparable to that of only Chk2 siRNA treatment (Figure 9c). As expected, control siRNA did not show any effect on G2–M arrest (Figure 9c). Further, we checked whether Chk2 siRNA used in the study was sufficient to suppress the expression of Chk2 protein by Western blotting in the similar treatment. The transfection with siRNA targeted to Chk2 suppressed the silymarin and silibinin (100 μg/ml for 24 h)-induced Chk2 protein expression (phospho as well as total) (Figure 9d). The expression of Chk2 was not affected in the cells transfected with a nonspecific control siRNA (Figure 9d). The effect of Chk2 down-regulation was also examined on silymarin- and silibinin-induced phosphorylation of Cdc25C, which showed a decrease in pCdc25C(Ser216) level in Chk2 siRNA-transfected cells (Figure 9d). Surprisingly, Chk2 siRNA transfection followed by silymarin and silibinin treatment resulted in more decrease in Cdc25C as compared to silymarin and silibinin treatments alone. At present the mechanism of such effect is not known, and needs further investigation. Overall, these results suggest that Chk2 plays a critical role in silymarin- and silibinin-induced G2–M arrest in PC3 cells.

Silymarin and silibinin induce phosphorylation of ATM (Ser1981) and histone 2AX (H2A.X) (Ser139)

ATM is an upstream kinase implicated in the phosphorylation and activation of Chk2 (Abraham, 2001). ATM is known to be activated by its autophosphorylation at Ser1981 in response to DNA damage, and also marked as an early response in such condition (Buscemi *et al*., 2004). Immunoblotting using an antibody specific for phospho-ATM(Ser1981) revealed an increased phosphorylation of ATM without any considerable change in its total protein level after 6 h of (100 μg/ml) silymarin and silibinin treatment (Figure 10a). Lysate from cells treated with 1 μM doxorubicin was used as a positive control, which also showed a remarkable increase in phospho-ATM(Ser1981) (Figure 10a). In response to

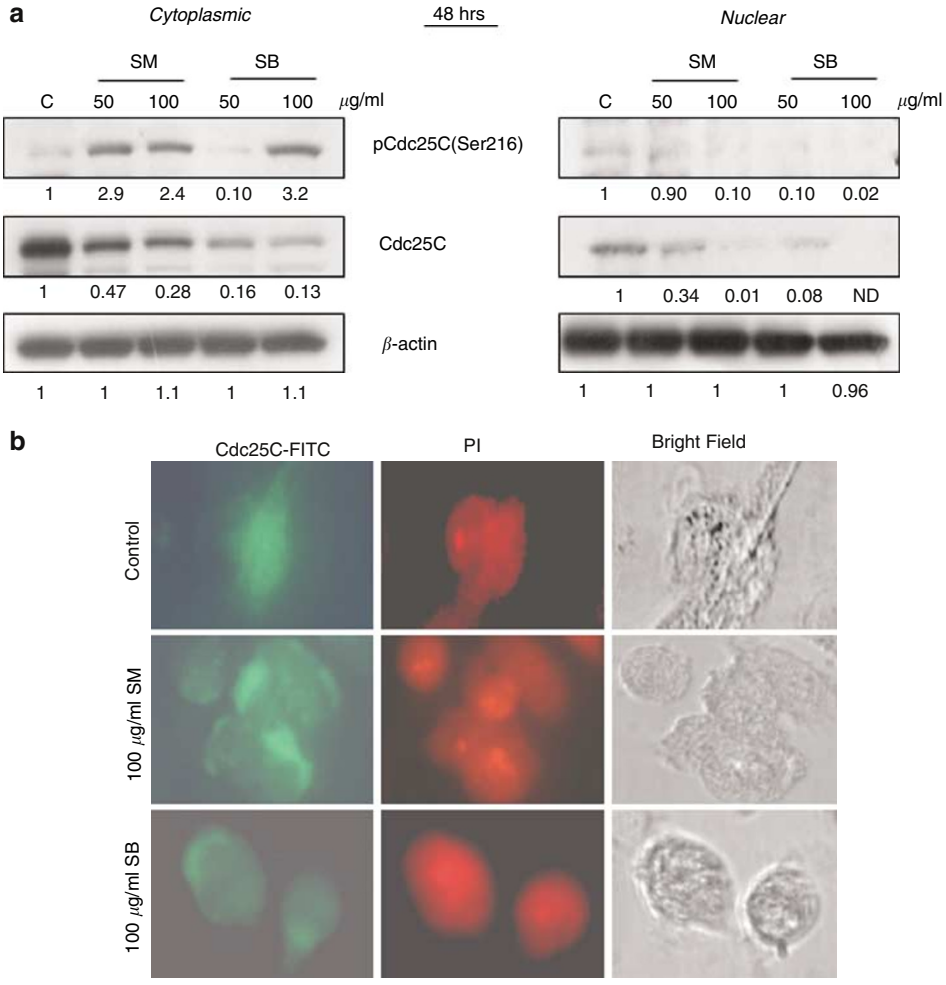

Figure 8 Effect of silymarin and silibinin on cytoplasmic and nuclear distribution of Cdc25C in PC3 cells. (**a**) Cells were treated as mentioned in Figure 6b, and cytoplasmic and nuclear fractions were analysed for pCdc25C(Ser216) and Cdc25C protein levels by Western immunoblotting as mentioned in 'Materials and methods', and membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. (**b**) Immunocytochemical analysis for cytoplasmic and nuclear distribution of Cdc25C in control (DMSO-treated) or 100 μg/ml dose of silymarin- or silibinin-treated cells. After 48 h of the treatment, cells were stained with anti-Cdc25C antibody (green) or propidium iodide (red) as described in 'Materials and methods'. A bright field picture of the same cells is shown in the third column. In control cells, Cdc25C was mainly localized in the nucleus, whereas, in silymarin- or silibinin-treated cells it was mainly localized around the nucleus and the cytoplasm also shows an overall decrease in green fluorescence as compared to control. Blots shown are representive of two independent experiments in each case. C, control; SM, silymarin; SB, silibinin; PI, propidium iodide; ND, not detectable.

DNA damage, ATM activation is known to phosphorylate H2A.X at Ser139, which has emerged as a sensitive marker for the presence of DNA double-strand breaks (Rogakou *et al*., 1998; Yih and Lee, 2000; Ye *et al*., 2001, 2004). Therefore, in similar treatments, we also examined the phosphorylation of H2A.X(Ser139) using phospho-specific H2A.X antibody, which was clearly increased after silymarin, silibinin, as well as doxorubicin treatments (Figure 10a). Protein loading was checked by immunoblotting the same membrane with anti-β-actin antibody (Figure 10a). Further, we carried out immunocytochemical analysis for the time-course phosphorylation of H2A.X(Ser139) in similar treatments. As shown in Figure 10b, a remarkable increase in phospho-H2A.X(Ser139) nuclear staining (green fluorescence) was observed as early as 6 h of silymarin, silibinin and doxorubicin treatments as compared to control (data for later time-points not shown). PI was used for nuclear staining (red fluorescence). Together, these results suggested the DNA-damaging effect of silymarin and silibinin leading to the activation of the ATM–Chk2 pathway for G2–M cell cycle arrest in human prostate carcinoma PC3 cells.

Discussion

Conventional chemo-/radio-therapy of advanced hormone-refractory PCA has done little to improve the treatment outcomes and quality of survival/life in human patients (Koivisto *et al*., 1998; Feldman and Feldman, 2001). Prevention and therapeutic intervention by

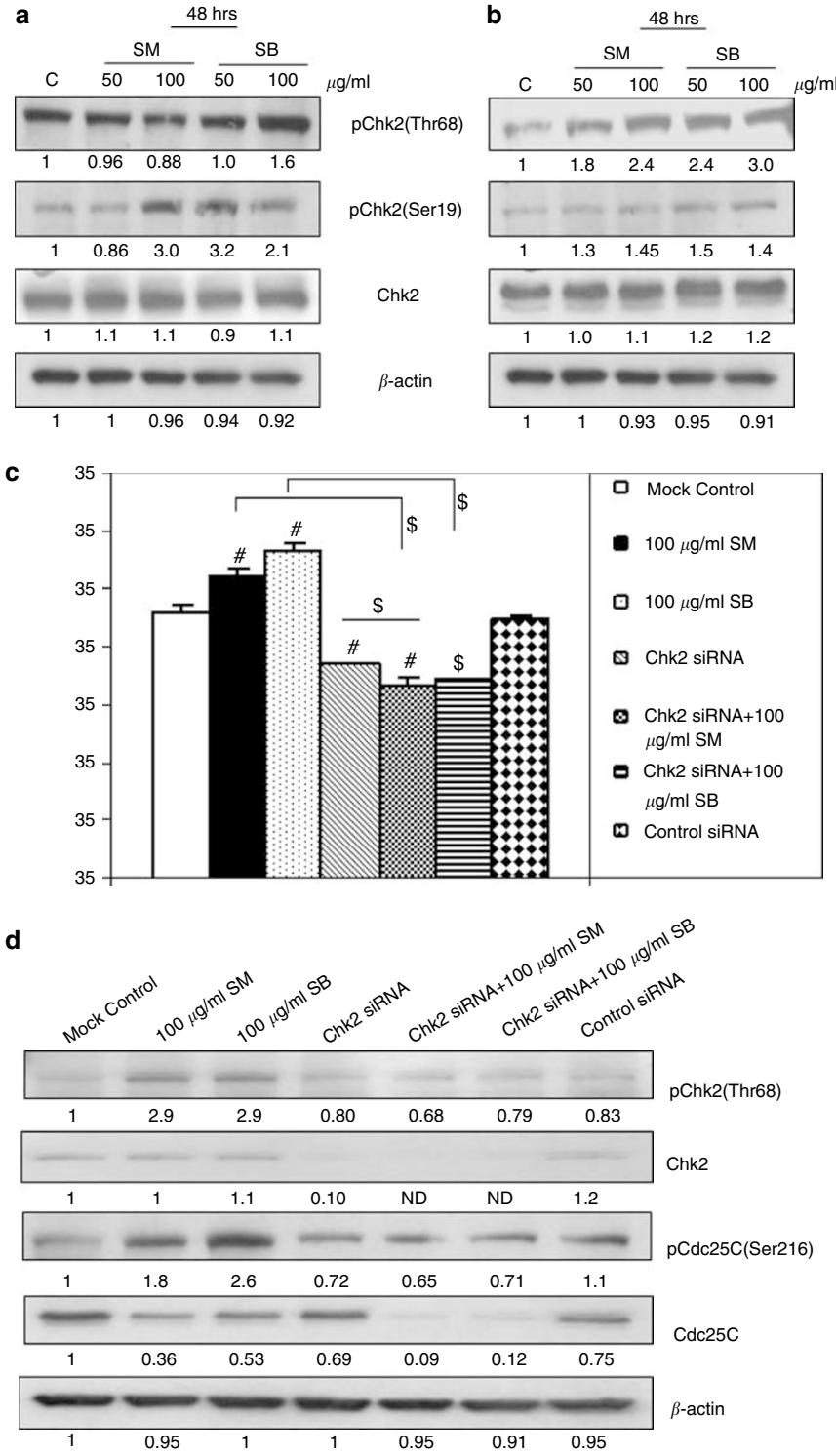

Figure 9 Effect of silymarin and silibinin on Chk2 activation and its role in mediating G2–M arrest in PC3 cells. (**a**, **b**) Cells were treated as mentioned in Figure 6a and b, and total cell lysates were prepared and analysed for pChk2(Thr68), pChk2(Ser19) and Chk2 protein levels by Western immunoblotting as mentioned in 'Materials and methods'. (**c**, **d**) Cells were mock transfected or transfected with Chk2 siRNA or control siRNA for 24 h as mentioned in 'Materials and methods'. Thereafter, cells were treated with DMSO or 100 mg/ml dose of silymarin or silibinin for 24 h. At the end of the treatment, total cells were collected and processed for (**c**) cell cycle analysis or (**d**) lysate preparation as mentioned in 'Materials and methods'. Cell lysates were analysed for pChk2(Thr68), Chk2, pCdc25C(Ser216) and Cdc25C protein levels by Western immunoblotting. (**a**, **b**, **d**) Membranes were also stripped and reprobed with anti-β-actin antibody for protein loading correction. In each case, blots shown are representive of three independent experiments. (**c**) Quantitative data shown for G2–M-phase cell population are representive of two independent experiments. C, control; SM, silymarin; SB, silibinin; $^{\#}P<0.05$; $^{\$}P<0.01$.

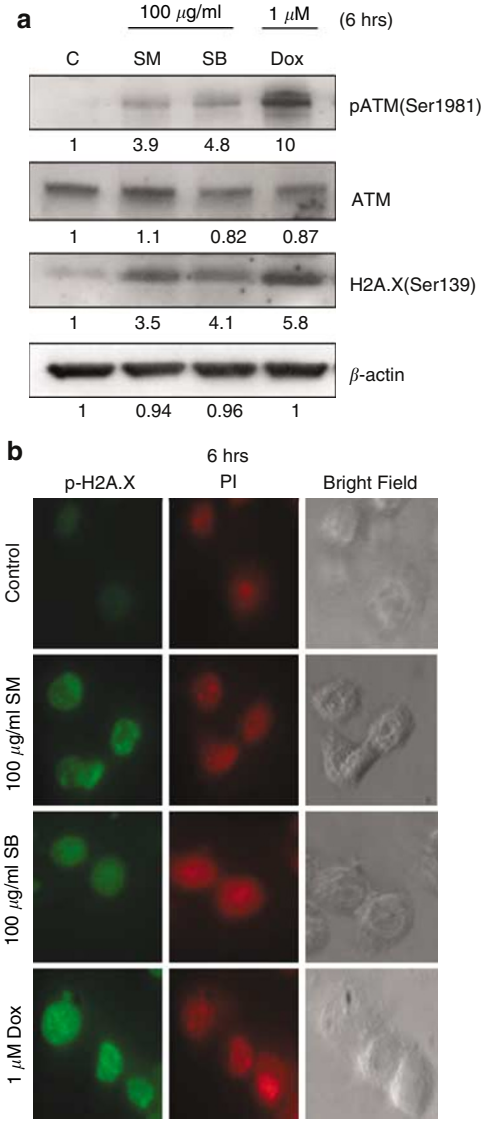

Figure 10 Effect of silymarin and silibinin on phosphorylation of ATM and H2A.X in PC3 cells. Cells were treated with DMSO (control) or 100 μg/ml dose of silymarin or silibinin or 1 μM doxorubicin for 6 h. (**a**) At the end of the treatments, cell lysates were prepared and analysed for pATM(Ser1981), ATM and pH2A.X(Ser139) by Western immunoblotting as mentioned in the 'Materials and methods'. Membrane used for immunoblotting of pH2A.X(Ser139) was stripped and reprobed for β-actin for protein loading correction. (**b**) In similar treatments, cells were immunocytochemically stained for pH2A.X(Ser139) (column 1, green fluorescence) and then counterstained with propidium iodide (column 2, red fluorescence) for nuclear staining as described in 'Materials and methods'. A bright field photograph of the same area is shown in column 3. Pictures are shown at ×200 magnification. Blots shown are representive of two independent experiments in each case. PI, propidium iodide; C, control; SM, silymarin; SB, silibinin; Dox, doxorubicin.

dietary and nondietary phytochemicals is a newer approach as compared to the use of cytotoxic chemotherapeutic agents in cancer management (Surh, 2003). Unchecked proliferative potential involving deregulation in cell cycle progression is generally described as a central process in the development of cancer (Hanahan and Weinberg, 2000). Therefore, one of the focuses in cancer-preventive strategies is to target the limitless replication characteristics and deregulated cell cycle progression in cancer cells (McDonald and El-Deiry, 2000; Owa *et al*., 2001). In this regard, the findings of the present study suggest that silymarin and silibinin exert strong antiproliferative effects against human prostate carcinoma PC3 cells, and that this effect involves alterations in cell cycle regulators, causing both G1 and G2–M arrests as well as cell death. These observations are supported, in part, by our earlier studies showing cell growth inhibition and G1 arrest by silymarin in human PCA DU145 cells (Zi *et al*., 1998b), and by silibinin in human PCA LNCaP cells (Zi and Agarwal, 1999). In the present study, we used human PCA PC3 cell line, which is mostly regarded as an intermediate cell type for aggressive behavior when compared with the DU145 and LNCaP cell lines. Furthermore, this is the first detailed comparative mechanistic study for the flavonolignan mixture silymarin and one of its major bioactive components, silibinin, for their cell cycle effects in human PCA cells.

Progression through the various phases of cell cycle is mediated by CDKs in complex with cyclins (Kastan *et al*., 1995). The activity of cyclin–CDK complexes is regulated at multiple levels such as expression, phosphorylation/dephosphorylation, proteolytic degradation, binding of inhibitors and subcellular localization (Obaya and Sedivy, 2002). Cyclin D-dependent kinases, CDK4 and CDK6, and cyclin E-dependent kinase CDK2 are largely involved in controlling G1–S restriction point (Grana and Reddy, 1995; Morgan, 1995; Sherr and Roberts, 1999). Cyclin D1 and cyclin D3 are known to bind with and activate CDK4, which phosphorylates Rb protein to release E2F transcription factors to transcribe genes needed for G1 to S transition (Sherr, 1995). Cyclin D1 is considered an oncogene, and overexpressed in many cancers, including prostate, breast, esophagus, lung, head and neck, and colon (Gillett *et al*., 1994; Adelaide *et al*., 1995; Bartkova *et al*., 1995; Arber *et al*., 1996; Caputi *et al*., 1999; Drobnjak *et al*., 2000). In the present study, silymarin and silibinin treatment to human PCA PC3 cells resulted in a decrease in cyclin D1, cyclin D3, CDK4 and CDK6 protein levels as well as kinase activity (Rb-GST phosphorylation) associated with CDK4. Further, silymarin and silibinin also altered the subcellular localization of cyclin D1, which was barely detectable in the nucleus at higher dose. All these molecular alterations by these agents would reduce the overall activity of this cyclin D1–CDK4 complex. Further, a noncatalytic role of cyclin D-dependent CDKs has also been reported, where they sequester Kip/Cip family of proteins in a mitogen-dependent manner, thereby facilitating the activation of CDK2–cyclin E complex (Sherr and Roberts, 1999). CDK2–cyclin E complex peaks at G1–S transition and phosphorylates several proteins, including Rb. In the present study, we observed that silymarin and silibinin decrease the protein levels of CDK2 and cyclin E, as well as nuclear localization of CDK2. As full activation of CDK2

requires its nuclear translocation, the cytoplasmic sequestration of CDK2 is expected to prevent cyclin E–CDK2 activation and reduce the overall activity of this complex. Consistent with these molecular alterations, both these agents decreased histone H1 kinase activity associated with CDK2. A similar mechanism has already been held responsible for the antiproliferative action of vitamin D in LNCaP cells (Yang and Burnstein, 2003). The inhibitory effect of silymarin and silibinin on these molecular parameters associated with G1–S transition could have led to the G1 arrest of PC3 cells as observed in cell cycle analysis.

The Cip/Kip family of CDKIs binds to and inhibits the activity of CDK–cyclin complexes that regulates G1–S and G2–M phase transitions (Deng *et al*., 1995; Agarwal *et al*., 2003; Nakayama *et al*., 2004; Takagaki *et al*., 2005). Cip1/p21 is a universal inhibitor of CDKs whose expression is normally regulated by the p53 tumor suppressor protein (Xiong *et al*., 1993; Grana and Reddy, 1995) as well as by p53-independent mechanisms (Zi *et al*., 1998b; Yim *et al*., 2005). Additionally, it plays a critical role in the cellular response to DNA damage for cell cycle arrest (Brugarolas *et al*., 1995). Kip1/p27 is another important member of CDKIs, which gets upregulated in response to antiproliferative signals for cell cycle arrest (Polyak *et al*., 1994). Reduced expression of Kip1/p27 protein is known as an independent prognostic marker in a large variety of cancers, and is associated with unfavorable prognosis (Lloyd *et al*., 1999; Langner *et al*., 2004). Our results showed that both silymarin and silibinin increase Cip1/p21 and Kip1/p27 protein levels. Further, in PC3 cells, the increase in CDKIs by both agents would be independent of p53 tumor suppressor protein as PC3 cells lack the p53 functional gene. We also observed that both the agents resulted in a marked decrease in the levels of Skp2 protein, which plays an important role in the degradation of both Cip1/p21 and Kip1/p27. Therefore, the increased levels of CDKIs by silymarin and silibinin might be partly due to decrease in the Skp2 level, and could be another mechanism to decrease CDK–cyclin kinase activity.

G2–M transition is regulated mainly by the sequential activation and deactivation of CDK-regulatory proteins and cyclin complexes (Taylor and Stark, 2001). Cdc2 is also known as CDK1, which initially forms a complex with cyclin A and later with cyclin B1 to drive the cell from G2 to M phase. Due to various genetic and epigenetic alterations, Cdc2 kinase activity is enhanced in many human cancers, allowing cell cycle progression of cancer cells having oncogenic mutations/defects in DNA for continued cell proliferation (Lloyd *et al*., 1999; Taylor and Stark, 2001). Therefore, induction of G2–M arrest in neoplastic cells is suggested as a promising approach to inhibit unchecked cell cycle progression and tumor growth. Silymarin and silibinin decreased Cdc2, cyclin A and cyclin B1 protein levels, with a concomitant decrease in Cdc2 kinase activity in PC3 cells. Further, levels of phosphorylated (inactive) and total Cdc2 in the nuclear and cytoplasmic compartments were altered by both of these agents in the favor of reduced activity of Cdc2. These molecular alterations by silymarin and silibinin could have led to a G2–M arrest in PC3 cells.

Cdc25 phosphatases control cell cycle progression by dephosphorylating and activating CDKs at positions Thr14 and Tyr15 (Gautier *et al*., 1991; Sebastian *et al*., 1993). Cdc25B is thought to function as a mitotic starter by dephosphorylating and activating CDK2/cyclin A and Cdc2/cyclin B (Nilsson and Hoffmann, 2000). Further, Cdc25C dephosphorylates and activates Cdc2/cyclin B mitotic kinase complex and thereby permits cell entry into mitosis (Turowski *et al*., 2003). Overexpression of dominant-negative mutants or microinjection of antibodies for Cdc25B and Cdc25C is shown to block the cell cycle progression at G2 phase (Millar *et al*., 1991; Seki *et al*., 1992; Gabrielli *et al*., 1996; Lammer *et al*., 1998). These reports suggest that Cdc25 phosphatases are critical regulators of Cdc2–cyclin B1 kinase activity. Consistent with these reports, silymarin and silibinin decreased both Cdc25B and Cdc25C protein levels, with a comparatively stronger effect on Cdc25C. Further, these agents also increased the phosphorylation of Cdc25C at Ser216, which is known to create a binding site for 14-3-3β proteins and export to and retention in the cytoplasm in the inactive form (Peng *et al*., 1997; Lopez-Girona *et al*., 1999; Singh SV *et al*., 2004). Consistent with a relative increase in Cdc25C(Ser216) phosphorylation, we also observed an increased binding of Cdc25C with 14-3-3β following silibinin and silymarin treatment. As expected, silymarin and silibinin treatment resulted in decrease in the levels of phospho- as well as total Cdc25C levels in the nucleus. Overall, these molecular changes could have favored the presence of an inactive form of Cdc2 that is phospho-Cdc2(Tyr15) for G2–M arrest, as observed in the present study.

Cdc25C phosphorylation is reported to be mediated via Chk2 kinase at Ser216 (Kawabe, 2004; Singh SV *et al*., 2004). Chk2, the mammalian ortholog of yeast Rad53/Cds1 kinase, is known to participate in cell cycle arrest in response to DNA damage (Buscemi *et al*., 2004). Genetic alterations of Chk2 have been identified in a wide spectrum of human sporadic tumors, including carcinomas of the breast, lung, vulva, colon, prostate and ovary, and osteosarcomas and lymphomas (Bartek and Lukas, 2003; Dong *et al*., 2003). In addition, germline mutations of Chk2 as well as its variants were found to be associated with familial carcinomas of the breast (Vahteristo *et al*., 2002) and prostate (Seppälä *et al*., 2003). The activation of Chk2 involves an initial phosphorylation step on Thr68 by ATM, the kinase that functions in DNA damage signaling by targeting several effector molecules, including Chk2 (Brown *et al*., 1999; Chaturvedi *et al*., 1999; Matsuoka *et al*., 2000; Melchionna *et al*., 2000). Consistent with these reports, silymarin and silibinin treatment resulted in increased phosphorylation of Chk2 at Thr68 and Ser19 sites. Downregulation of Chk2 using siRNA resulted in abolition of silymarin- and silibinin-induced G2–M arrest, as well as decreased the phosphorylation of Chk2 at Thr68 and Cdc25C at Ser216 sites. Surprisingly, we also observed a decrease in the Cdc25C protein level;

the reason for this effect is not known at present and needs further investigation. These findings suggested the central role of Chk2 in silymarin- and silibinin-induced G2–M arrest.

ATM is a member of the phosphoinositide 3-kinase-like family of serine/threonine protein kinases and plays a critical role in cellular response to DNA damage such as cell cycle arrest, DNA repair and apoptosis induction (Abraham, 2001; Buscemi *et al*., 2004). Activation of ATM results in the phosphorylation of a diverse array of downstream targets that participate in numerous cellular events, including regulation of three cell cycle checkpoints (G1, intra-S and G2–M) and apoptosis (Abraham, 2001). In addition to activating Chk1 and Chk2 for cell cycle arrest, ATM is known to phosphorylate a histone variant protein H2A.X at Ser139, marking a double-strand DNA break and initiation of apoptosis (Abraham, 2001; Zhang *et al*., 2002; von Zglinicki *et al*., 2005). Consistent with these reports, silymarin and silibinin resulted in increased phosphorylation of ATM at Ser1981 (activated form), which could have led to the increased phosphorylation of Chk2(Thr68) mediating G2–M cell cycle arrest, and H2A.X(Ser139) for the onset of apoptotic cell death in PC3 cells. The increased phosphorylation of ATM(Ser1981) as well as H2A.X(Ser139) also indicates DNA double-strand breaks by silymarin and silibinin in PC3 cells to decrease cell survival; however, neither silymarin not silibinin have been reported previously to cause DNA damage in PCA cells. Therefore, the identification as well as mechanism/s for DNA damage needs further investigation.

In summary, silymarin and silibinin induced G1 and G2–M cell cycle arrest involving molecular alterations in cell cycle regulatory proteins in human prostate carcinoma PC3 cells. G1 arrest appears to be mediated via a decrease in CDK4, CDK6, CDK2, cyclin D1 and cyclin D3 protein levels, ultimately leading to the decrease in kinase activity of these G1-phase CDKs. Cytoplasmic retention of cyclin D1 and CDK2, and increased levels of Cip1/p21 and Kip1/p27 by silibinin and silymarin, could constitute additional mechanisms to inhibit the kinase activity of G1-phase CDKs. Similarly, G2–M arrest was mediated via decrease in the Cdc2 kinase activity, involving a decrease in the protein levels of Cdc2, cyclin A and cyclin B1. Further decrease in the levels of Cdc25B and Cdc25C, accompanied by an increased phosphorylation of Cdc25C(Ser216) resulting in its translocation to cytoplasm and binding with 14-3-3β, could have a role in the G2–M arrest. Increased Chk2 phosphorylation at Thr68 was critical in silymarin- and silibinin-induced G2–M arrest. Further, upstream ATM activation could have led to Chk2 activation and phosphorylation of H2A.X(Ser139), contributing to G2–M arrest and decrease in cell survival, as observed in the present study. It should be noted that most of the results obtained from silymarin and silibinin were comparable, suggesting silibinin as a major component in flavonolignan mixture silymarin causing cell cycle arrest in PC3 cells. However, some effects were more pronounced with silymarin, such as the total and cytoplasmic levels of Cdc25C(Ser216) phosphorylation (Figures 7a (24 h treatment) and 8a), while silibinin was more effective on suppressing total and Tyr15-phosphorylated Cdc2 levels (Figure 6a and b). These findings necessitate the further identification, isolation and investigation of other isomers of silibinin present in silymarin for their anticancer efficacy and associated mechanisms. In this direction, studies are in progress in our laboratories to isolate silibinin isomers from silymarin and assess their anticancer efficacy and associated mechanisms in different human PCA cells. We expect that ongoing studies will lead to the identification of new anticancer components present in silymarin other than silibinin.

Materials and methods

Cell line and reagents

Human prostate carcinoma PC3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Silymarin and silibinin were from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). RPMI1640 and other culture materials were from Invitrogen Corporation (Gaithersberg, MD, USA). Antibodies to Cip1/p21 and phospho-H2A.X(Ser139) were from Upstate (Charlottesville, VA, USA), and to Kip1/p27 were from Neomarkers, Inc. (Fremont, CA, USA). Antibodies for total CDK2, CDK4, CDK6, Cdc2, Cdc25A, Cdc25B, Cdc25C, Wee1, Chk2, cyclin D1, cyclin D3, cyclin E, cyclin B1, 14-3-3β and Skp2, and RB-GST fusion protein were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for phospho-Cdc2, Cdc25C and Chk2 were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibody for β-actin, and propidium iodide and doxorubicin were from Sigma. Antibody for phospho-ATM (Ser1981) and total ATM was from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). Histone H1 was from Boehringer Mannheim Corp. (Indianapolis, IN, USA). Alexa Fluor 488 goat anti-mouse IgG conjugate was from Molecular Probes (Eugene, OR, USA). Goat anti-rabbit FITC conjugate was from Santa Cruz Biotechnology. A/G-plus agarose and A-plus agarose beads were from Santa Cruz Biotechnology, Inc. [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) and ECL detection system were from Amersham Corporation (Arlington Heights, IL, USA). Chk2 siRNA was from Santa Cruz Biotechnology. *Trans*IT-TKO transfection reagent was from Mirus Bio Corporation (Madison, WI). Other chemicals were obtained in their commercially available highest purity grade.

Cell culture and treatments

PC3 cells were cultured in RPMI1640 medium with 10% fetal bovine serum and 100 U/ml penicillin G–100 μg/ml streptomycin sulfate under standard mammalian cell culture conditions. Cells were then treated with different doses of silymarin and silibinin (50 and 100 μg/ml concentrations in medium) dissolved in DMSO for the desired time periods (24–48 h) in serum condition. An equal amount of DMSO (vehicle) was present in each treatment, including control. At the end of each treatment time, whole-cell lysates or cytosolic and nuclear extracts were prepared as described previously (Dhanalakshmi *et al*., 2002).

Cell growth and death assays

PC3 cells were plated at 5000 cells/cm^2, and after 24 h, fed with fresh medium and treated with different doses of silymarin and

silibinin (50 and 100 μg/ml concentrations in medium). After 24 and 48 h of these treatments, total cells were collected by brief trypsinization, and washed with PBS. Total cell number was determined by counting each sample in duplicate using a hemocytometer under an inverted microscope. Cell viability was determined using trypan blue exclusion method. Each treatment and time point had three independent plates. The data shown in this study are the mean of three independent experiments.

Fluorescence-activated cell sorting (FACS) analysis for cell cycle distribution

PC3 cells were treated with desired doses of silymarin and silibinin (50 and 100 μg/ml concentrations) in complete medium for 24 and 48 h. At the end of each treatment time, cells were collected after a brief incubation with trypsin-EDTA, followed by processing for cell cycle analysis as reported earlier (Agarwal *et al*., 2003). Briefly, 0.5×10^5 cells were suspended in 0.5 ml of saponin/PI solution (0.3% saponin (w/v), 25 μg/ml PI (w/v), 0.1 mM EDTA and 10 μg/ml RNase A (w/v) in PBS), and incubated overnight at 4°C in dark. Cell cycle distribution was then analysed by flow cytometry using FACS analysis core facility of the University of Colorado Cancer Center.

Western immunoblotting

At the end of the desired treatment, cell lysates or nuclear and cytosolic extracts were prepared in nondenaturing lysis buffer as recently reported by us (Dhanalakshmi *et al*., 2002). As needed, 40–60 μg of protein lysates per sample was denatured in 2× SDS–PAGE sample buffer and subjected to SDS–PAGE on 6, 12 or 16% tris-glycine gels as needed. The separated proteins were transferred onto nitrocellulose membrane, followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. Membranes were probed for the protein levels of desired molecules using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody, and visualized by ECL detection system. Membranes were stripped and reprobed with anti-β-actin antibody to check the equal protein loading.

Immunoprecipitation and kinase assays

CDK2- and Cdc2-associated H1 histone kinase activity was determined as described by us recently (Agarwal *et al*., 2003) with some modifications. Briefly, 200 μg of protein lysates from each sample was precleared with protein A/G-plus agarose beads, and CDK2 and Cdc2 protein were immunoprecipitated using anti-CDK2 and Cdc2 antibodies (2 μg/sample) and protein A/G-plus agarose beads. The beads were washed three times with lysis buffer and finally once with kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 30 μl of 'hot' kinase solution (2.5 μg of histone H1, 0.5 μl of [γ-^{32}P]ATP, 0.5 μl of 0.1 mM ATP and 28.75 μl of kinase buffer) for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were resolved on 12% SDS–PAGE, and the gel was dried and subjected to autoradiography.

Similarly, to determine the CDK4-associated retinoblastoma (Rb) kinase activity, CDK4 protein was immunoprecipitated using specific antibody, and agarose A/G-plus beads, conjugated antibody beads and proteins were washed three times with Rb-lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 80 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 0.1% Tween 20, 10% glycerol, 1 mM PMSF, and 10 μg/ml leupeptin and aprotinin) and once with Rb kinase assay buffer (50 mM HEPES-KOH, pH 7.5, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate and 1 mM DTT). Phosphorylation of Rb was measured by incubating the beads with 30 μl of 'hot' Rb kinase solution (2 μg of Rb-GST fusion protein, 0.5 μl of γ-^{32}P-ATP, 0.5 μl of 0.1 mM ATP and 28.75 μl of Rb kinase buffer) for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. Samples were resolved on SDS–PAGE, and the gel was dried and subjected to autoradiography.

Cdc25C and 14-3-3β *binding study*

Cells were treated with DMSO or 100 μg/ml dose of silymarin or silibinin for 6 h, and cell lysates were prepared. Equal amount of protein lysates from each sample was precleared with protein A agarose beads, and 14-3-3β protein were immunoprecipitated using anti-14-3-3β antibody. Beads complexed with antibody and protein were washed thrice with lysis buffer, and boiled with 2× sample buffer and resolved on 12% tris-glycine gel and transferred onto a nitrocellulose membrane. The membrane was first probed with anti-Cdc25C antibody, and again stripped and reprobed with anti-14-3-3β antibody as mentioned above.

Microscopic analysis for nuclear/cytoplasmic distribution of Cdc25C

PC3 cells (2×10^4) were grown on coverslips and allowed to attach overnight. Cells were then exposed to DMSO or silymarin and silibinin (100 μg/ml) for 48 h at 37°C, washed with PBS, and fixed with 4% paraformaldehyde for 30 min at room temperature. After blocking for 45 min with normal goat serum, cells were incubated with anti-Cdc25C antibody (1:200 dilution with PBS containing 1% bovine serum albumin) for 1 h at room temperature. After washing, cells were treated with Alexa Fluor 568 secondary antibody and counterstained with PI. Cells were then washed with PBS six times and observed and photographed under an inverted Nikon TE-300 microscope with an epifluorescent attachment equipped with a Princeton Instrument Micromax camera, at 488 nm fluorescence excitation and 520 nm fluorescence emission. Images were acquired with Image Pro-plus software (Media Cybernetics, Silver Spring, MD, USA) at ×200 magnification.

Chk2 siRNA transfection

RNA interference of Chk2 was performed using 21-bp (including a 2-deoxynucleotide overhang) siRNA duplexes. For transfection, PC3 cells were seeded in 60 mm plates and transfected at 40% confluence with 200 nM siRNA duplexes using *Trans*IT-TKO transfection reagent according to the manufacturer's recommendations. Cells were treated with *Trans*IT-TKO transfection reagent (mock) or transfected with a nonspecific siRNA duplex as control for direct comparison. After 24 h of transfection, cells were treated with DMSO or silymarin/silibinin for 24 h. Both floating and adherent cells were collected, washed with PBS, and processed for analysis of cell cycle distribution or cell lysate preparation for immunoblotting as mentioned above.

Immunocytochemical staining for phospho-H2A.X(Ser139)

PC3 cells were seeded on four-well chamber slides (2×10^4 cells/chamber) and the next day treated with DMSO (control) or 100 μg/ml silibinin and silymarin or 1 μM doxorubicin (positive control). At the end of the desired treatment times, cells were fixed with methanol at −20°C for 10 min and washed

twice with ice-cold PBS. Cells were gradually rehydrated with PBS and then incubated with 10% BSA in PBS for 30 min at room temperature. Cells were rinsed twice with PBS and incubated with primary mouse monoclonal anti-phospho H2A.X(Ser139) antibody in PBS with 3% BSA at 4°C overnight. Cells were then rinsed in PBS with 3% BSA six times and incubated with Alexa fluor 488-conjugated goat anti-mouse IgG secondary antibody for 1 h. Cells were then rinsed in PBS with 3% BSA six times and observed and photographed as mentioned above for Cdc25C staining.

Statistical analysis

Statistical analysis was performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA, USA) as needed. Data were analysed using *t*-test as needed and a statistically significant difference was considered to be at $P<0.05$. For all the results where applicable, the autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA), and the mean density of each band was analysed by the Scion Image program (NIH, Bethesda, MD, USA). In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. Densitometry data presented below the bands are 'fold change' as compared to control for both silymarin and silibinin treatments in each case.

Abbreviations

PCA, prostate cancer; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; Cdc25C, cell division cycle 25C; Chk, checkpoint kinase; H2A.X, histone 2AX; FACS, fluorescence-activated cell sorting; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia-Rad3-related.

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